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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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WILMER CUTLER PICKERING HALE AND DORR LLP
60 STATE STREET
BOSTON, MA 02109

EXAMINER	
YAO, LEI	

ART UNIT	PAPER NUMBER
1642	

DATE MAILED: 11/02/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/736,889	GEORGES ET AL.	
	Examiner	Art Unit	
	Lei Yao, Ph.D.	1642	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *07 August 2006*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-108* is/are pending in the application.

4a) Of the above claim(s) *11,13,20-58,67,69 and 75-108* is/are withdrawn from consideration.

5)☒ Claim(s) *1-9 and 59-65* is/are allowed.

6)☐ Claim(s) *10, 12, 14-19, 66, 68 and 70-74* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *12/15/03* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☒ Other: *exhibit A, B*.

RESPONSE TO ARGUMENTS

The Amendment filed on 8/7/06 in response to the previous Non-Final Office Action (5/3/06) is acknowledged and has been entered.

Claims 1-108 are pending. Claims 11, 13, 20-58, 67, 69, 75-108 have been withdrawn previously for non-elected invention. Claims 1-9 and 59-65 have been allowed and claims 10, 12, 14-19, 66, 68 and 70-74 have been rejected.

The text of those sections of Title 35, U.S.Code not included in this action can be found in the prior Office Action.

Rejections Withdrawn

1. The objection of the specification because it contains embedded hyperlinks is withdrawn in view of the amendments to the specification by deleting the embedded hyperlinks.

Response to Arguments

Rejection under 35 USC § 103

1. Claims 10, 12 and 14-19 remain rejected under 35 U.S.C. 103(a) as being unpatentable over Meschini et al., in view of Fanger et al., and Heidenthal et al., as stated below:

> Meschini et al., teach a method of measuring the expression of vimentin in Multidrug Resistant (MDR) neoplastic cells. Meschini et al., first teach that drug resistant cells express MDR protein, p-glycoprotein, (page 620, page 4 and page 621, figure 5). Meschini et al., then, teach that the resistant cells display a high level of vimentin by flow cytometry analysis and immunocytochemical staining, which are correlated with MDR protein expression, while the drug sensitive cells express very low levels of vimentin (page 618, col 1, table II and page 619, figure 2). Meschini et al., further teach the expression of vimentin is determined by antibody to vimentin and measured by immunofluorescence emission (page 618, Table II and 619, figure 2). Although Meschini et al., do not specifically teach that expression of vimentin is on the cell surface of MDR neoplastic cells, figure 2 of the immunocytochemical staining of vimentin does suggest the surface expression of vimentin on the MDR cells.
>
> Meschini et al., do not teach detecting MDR cells by modified LDL, a vimentin binding agent, linked to a detectable agent and detecting MDR by administering the vimentin binding agent.
>
> Fanger et al., teach that administering LDL or AcLDL (modified LDL) to a patient, (column 3, para 1). Fanger et al., also teach the LDL is fluorophores-labeled or radiolabeled, which bind to the cell expressing LDL binding protein on the surface in vivo (column 3 para 2 and column 11, para 1).
>
> Heidenthal et al., teach that modified LDL binds to vimentin (entire article).
>
> It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to detect the MDR cells in a patient by measuring the level of modified LDL administered and bound to the vimentin expressed on the surface of the MDR cells in a patient. One of ordinary skill in the art would have been motivated with a reasonable expectation of success to combine the

teachings of Fanger et al., and Heidenthal et al., to Meschini et al., to detect the MDR cells by detecting the levels of labeled modified LDL binding on the surface of the MDR cells in a patient because Meschini et al., has suggested that a high level of vimentin comprising surface expressed vimentin is detected in the MDR cells compared to the drug sensitive cells, Fanger et al., has shown that administered labeled LDL binds to the cells expressing LDL binding protein and Heidenthal et al., has shown modified LDL binds to vimentin. One of ordinary skill in the art would have been motivated with a reasonable expectation of success to replace the antibody to vimentin taught by Meschini et al., with the modified LDL taught by Heidenthal et al., and determine MDR cells by measuring the levels of the modified LDL on the cell surface.

The response filed 8/7/06 has been carefully considered but is deemed not to be persuasive. The response states that *claim 10 is directed to a method for detecting a multidrug resistant (MDR) cell in a patient in which cell-surface expressed vimentin is detecting by a vimentin binding agent, Meschini teach vimentin expressed by MDR cells, but does not teach or suggest the detection of cell-surface-expressed vimentin and both Fanger and Heidenthal do not teach to suggest detecting of cell-surface expressed vimentin.* In response to this argument, first, the set of the claims, as written, are directed to an in vivo method for detecting a MDR in a patient by administering and detecting a labeled vimentin binding agent binding to vimentin expressed on the surface of the MDR cells. One skilled in the art would interpret that applicants are claiming a method of determining MDR in a patient through detecting a vimentin binding agent, not vimentin itself, while MDR cell expressing vimentin is merely a necessary and required factor for the agent to bind. Heidenthal et al., have shown vimentin binding agent, modified LDL, Fanger et al., have shown administrating labeled LDL to a patient for the purpose of detecting the cells that bind to, and Meschini et al., have shown that detection of vimentin in all location of MDR cells comprising cell surface (see figure 2), which would allow the modified LDL binding to and being detected together with MDR cells. Because the claim limitations are all taught by three references, it would be obvious for one skilled in the art to combine the teachings of all tree references to use the method to determine whether a patient is drug resistant in the clinic. One of ordinary skill in the art would have been motivated with a reasonable expectation of success to use the method in order to get a benefit of rapid detection of MDR during the chemotherapeutic treatment of a patient.

Applicants then argue that *Meschini et al., teach away from the claimed invention because detection of protein expressed only in the cell interior is explicitly taught in the reference (page 4-5).* In response to this argument, Meschini et al., teach that MDR is associated with expression of vimentin and

a method of detecting vimentin. Meschini et al., do not teach that the vimentin is only located intracellularly, instead, Meschini et al., do show a method of detecting vimentin expressed on the surface of the cell. For example, on page 616, column 2, paragraph 3, Meschini et al., describe a method for detecting vimentin in cell suspension fixed in 2% paraformaldehyde in PBS by immunocytochemistry staining with an antibody. One skilled in the art clearly knows that the cell fixed in such condition is good for detection of membrane antigen (on the surface of the cells) by an antibody as evidenced by a standard protocol for immunohistochemistry (exhibit A). One skilled in the art also knows that such fixation is not a condition for permeabilizing cells for the detection of the intracellular antigen. Thus, one skilled in the art would understand and realize that the visualized vimentin in the cells fixed in such condition and stained by fluorescent-labeled antibody in figure 2 of Meschini would be on the surface of the MDR cells. Applicants again argue the teachings by Heidenthal et al., and Fanger et al., which have been discussed above.

Thus, applicant's arguments have not been found persuasive, and the rejection is maintained.

2. Claims 66, 68 and 70-74 remain rejected under 35 U.S.C. 103(a) as being unpatentable over Thomas et al., in view of Fanger et al., and Heidenthal et al. as stated below:

> Thomas et al., teach the vimentin expression in the cells of breast cancer samples from breast cancer patients (page 2701, column 1, para 1). Thomas et al., teach a method of detecting vimentin expression determined by antibody for vimentin labeled with fluorescence dye (Rhodamine, figure 3, page 2701). Thomas et al., also teach that the method can be used for diagnosis of breast pathology and poor prognosis (page 2699, column 1, line 12-13). Although Thomas et al., do not specifically teach that expression of vimentin is on the cell surface of neoplastic cells, the figure 1 and 3, histological staining of vimentin on the breast cancer tissues do suggest that the expression of vimentin comprises the surface expression of the protein on the cells.
>
> Fanger et al., teach that administering LDL or AcLDL (modified LDL) to a patient, (column 3, para 1). Fanger et al., also teach the LDL is fluorophores-labeled or radiolabeled, which bind to the cell expressing LDL binding protein on the surface in vivo (column 3 para 2 and column 11, para 1).
>
> Heidenthal et al., teach that modified LDL binds to vimentin (entire article).
>
> It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to detect neoplastic cells in a patient by measuring detectable levels of the modified LDL administered to a patient and bound to vimentin expressed on the neoplastic cells from the patient. One of ordinary skill in the art would have been motivated with a reasonable expectation of success to combine the teachings of Fanger et al., and Heidenthal et al., to teaching of Thomas et al., to detect the neoplastic cell by detecting the modified LDL bound to the surface expressed vimentin on the cells because Thomas et al., have shown that vimentin is expressed in the neoplastic cell and the figures disclosed by the reference has suggested the expression of vimentin is detected on the surface of the cells, and because Fanger et al., have shown that administered labeled LDL binds to the cells expressing LDL binding protein and Heidenthal et al., has shown that binding of modified LDL to vimentin.

The response filed 8/7/06 has been carefully considered but is deemed not to be persuasive. Applicants argue that Thomas et al., teach detection of vimentin in intermediate filaments, which exist within cells and cite a book (Molecular Biology of the Cell) by Albers et al., to support intermediate filaments located within cells (page 6-7). In response to this argument, Thomas et al., teach that vimentin as an intermediate filament protein is expressed in the progressive breast cancer. Thomas et al., do not specifically teach that the detectable vimentin in the breast cancer tissue is within the cells, instead, in figure 3, Thomas et al., clearly shows that the vimentin is located on the cell surface. Although, as an intermediate filament protein, vimentin play a role for connecting nuclei and cell membrane, many evidences have been shown by one skilled in the art that vimentin is detectable on the cell surface, especially, on the abnormally growing or grown cells under the stress condition as evidenced by Mosan et al., who teach that detectable surface vimentin on the human neutrophil (J of leuk Bio, vol 79, page 1-10, exhibit B). The Applicants again argue the references by Fanger et al., and Heidenthal et al., for the same reasons, which have been discussed above. Thus, applicant's argument has not been found persuasive, and the rejection is maintained.

Conclusion:

Claims 10, 12 and 14-19 66, 68 and 70-74 remain rejected. Claims 1-9 and 59-65 have been allowed.

Meschini et al., (Int, J. Cancer, Vol 87, page 615-628, 2000) teach that MDR cells express vimentin and show a method of staining vimentin in the cells. Meschini et al., do not teach or suggest a method of detecting MDR by measuring cell surface-expressed vimentin.

THIS ACTION IS MADE FINAL. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of

the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Lei Yao, Ph.D. whose telephone number is 571-272-3112. The examiner can normally be reached on 8am-6.00pm Monday-Thursday.

Any inquiry of a general nature, matching or file papers or relating to the status of this application or proceeding should be directed to Kim Downing for Art Unit 1642 whose telephone number is 571-272-0521

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Jeffrey Siew can be reached on 571-272-0787. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Lei Yao, Ph.D.
Examiner
Art Unit 1642

LY

JEFFREY SIEW
SUPERVISORY PATENT EXAMINER

IOWA STATE UNIVERSITY Flow Cytom
of

INSTRUMENTATION

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Indirect Labeling

Fixation

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Exhibit A

Paraformaldehyde Fixation of Cells

Background

This fixation method is good for cells labeled by fluorochrome-conjugated antibodies to membrane antigens. It will stabilize the light scatter and labeling for up to a week in most instances, allowing you to be more flexible in scheduling cytometer time. Furthermore, it inactivates most biohazardous agents, so it is important from a safety standpoint as well.

The procedure picks up at the end of the direct or indirect staining procedures. The cells are expected to be in 12 x 75 mm. plastic culture tubes, one million cells per tube.

It should not be used with the procedure to label dead cells. Fixed cells have a permeable membrane - the dye would enter *all* the cells.

Materials

1. **2X Paraformaldehyde Stock Solution.**
   - Add 2 g. paraformaldehyde to 100 ml. PBS+azide.
   - Heat to 70 degrees Celsius in a fume hood, or in a 56 degree Celsius water bath, just until the paraformaldehyde goes into solution.
   - Allow to cool to room temperature, then adjust to pH 7.4 using 0.1 M. NaOH or 0.1 M. HCl, as needed.
   - Store at 4 degrees Celsius.

2. **0.5% Paraformaldehyde Working Solution.** Add 10 ml. of the 2% Stock Solution to 30 ml. PBS+azide. Store at 4 degrees Celsius. This solution is stable for up to 1 week.

3. **Antibody-labeled cells in PBS+azide.** They may

Iowa State University
Flow Cytometry
Facility
1104 Molecular
Biology Building
Ames, IA 50011
515-294-2472

have been labeled using either the direct or indirect labeling procedures. Concentration should be 1 million cells in 1 ml.

Equipment

1. **pH meter.** This is involved in making the paraformaldehyde stock solution.
2. **Balance** with a resolution of at least 0.1 g. Again, this is to make the paraformaldehyde stock solution.
3. **One liter graduated cylinder or volumetric flask.** *Ditto.*
4. **Centrifuge.** You should know how the RPM translates into G-force.
5. **Pipette** in the range of 500 to 1000 microliters (0.5-1.0 ml.).
6. **Vortex mixer.** You *could* mix by tapping or shaking the tubes, but a mixer will give much more reproducible results in most cases.
7. **Refrigerator.** To store the preserved cells.

Procedure

1. Following the last wash step, centrifuge the cells and remove the liquid, as described in the direct or indirect antibody labeling procedure.
2. Add 0.5 to 1.0 ml. of cold 0.5% paraformaldehyde solution. **Vortex immediately.**
3. Store the cell suspension at 4 degrees Celsius in the dark.

Analyze the cells on the flow cytometer within one week.

Protocol adapted from University of Florida Flow Cytometry Core

Cell surface expression of intermediate filament proteins vimentin and lamin B_1 in human neutrophil spontaneous apoptosis

Eliane Moisan and Denis Girard[1]

INRS-Institut Armand-Frappier, Université du Québec, Canada

Exhibit B

Abstract: Neutrophils represent an important source of autoantigens for antineutrophil cytoplasmic antibody associated with vasculitis. To date, two cytoskeletal proteins, vinculin and vimentin, have been reported to be expressed on the cell surfaces of activated macrophages, platelets, and apoptotic T lymphocytes. However, such cell surface expression has never been studied in human neutrophils. As we recently demonstrated that different cytoskeletal proteins were cleaved in apoptotic neutrophils, we hypothesized that some of these were expressed on the cell surface of apoptotic neutrophils. Herein, we found that among vinculin, paxillin, gelsolin, vimentin, lamin B_1, α-tubulin, and β-tubulin, only the two intermediate filament (INFIL) proteins, vimentin and lamin B_1, are expressed on the cell surface of 24-h aged neutrophils [spontaneous apoptosis (SA)]. By monitoring intracellular expression of vimentin and lamin B_1 during SA, we found that these two proteins were cleaved and that such cleavage was reversed by the pan caspase inhibitor N-benzyloxycarbonyl-V-A-D-O-methylfluoromethyl ketone (z-VAD-fmk). When neutrophil apoptosis was delayed or suppressed by lipopolysaccharide or the cytokines granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage (GM)-CSF, or interleukin-4, the loss of intracellular expression of vimentin and lamin B_1 was prevented. The INFIL proteins were absent from the cell surface when neutrophil apoptosis was delayed. Addition of z-VAD-fmk significantly decreased the cell surface expression of vimentin and lamin B_1 during SA. This study provides the first evidence that apoptotic neutrophils express cytoskeletal proteins on their surface, opening the possibility that these cells may participate in the development of autoantibodies directed against cytoskeletal proteins, a condition frequently reported in several inflammatory diseases. *J. Leukoc. Biol.* 79: 000–000; 2006.

Key Words: *cytoskeleton · microfilaments · microtubules · caspases · flow cytometry*

INTRODUCTION

The cytoskeleton is a dynamic structure composed of numerous proteins, which form networks. Several neutrophil functions require rearrangement of the cytoskeleton, including chemotaxis, phagocytosis, degranulation, and apoptosis [1]. The cytoskeleton may be involved in many autoimmune disorders, as anticytoskeletal autoantibodies can be found in patients suffering from various rheumatic diseases [2–5]. However, the origin of these autoantibodies is still unclear. It was recently found that vinculin, a microfilament-associated protein (MFAP), was expressed on the surface of apoptotic T lymphocytes [6]. The intermediate filament (INFIL) protein vimentin was found on the surface of activated platelets, where it was bound to vitronectin and plasminogen activator inhibitor complexes [7]. Recently, Boilard et al. [8] showed that secreted human group IIA phospholipase A_2 (PLA_2) binds to vimentin on the cell surface of apoptotic T lymphocytes. The interaction between these two proteins enhanced the activity of PLA_2, suggesting that vimentin may play a role in PLA_2-mediated cellular arachidonic acid release. Mor-Vaknin et al. [9] demonstrated that stimulation of human macrophages activated phosphorylation of vimentin, leading to its expression on the cell surface and its secretion into the extracellular milieu. Furthermore, they showed that extracellular vimentin played a role during inflammation, as it acted as a protein involved in killing bacteria and in oxidative metabolite production; this activity was enhanced by proinflammatory cytokines [9]. In addition, a population of endothelial cells was found to express vimentin on the cell surface and to secrete into bloodstream [10]. Knowing that some cytoskeletal proteins are implicated in inflammatory functions, these observations can additionally explain, in part, the presence of anticytoskeletal autoantibodies found in many autoimmune diseases.

Apoptotic cells are an important source of autoantigens [11]. Two studies have demonstrated that injection of apoptotic cells into normal mice resulted in production of various autoantibodies [12, 13], including antivimentin autoantibodies [13]. Aside from erythrocytes and platelets, neutrophils are the predominant cells in the circulation, and because of this, in addition to the fact that these cells are known to undergo apoptosis spontaneously [14, 15], they represent an important source of cytoskeletal autoantigens. Recently, we have demonstrated that certain cytoskeletal proteins, including vimentin, are cleaved during spontaneous or agent-induced human neutrophil apoptosis [16–19]. Therefore, we hypothesized that

[1] Correspondence: INRS-Institut Armand-Frappier, 245 boul. Hymus, Pointe-Claire (PQ), Canada, H9R 1G6. E-mail: denis.girard@iaf.inrs.ca

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degradation of cytoskeletal proteins in neutrophils could lead to their cell surface expression.

In the present study, we investigated potential cell surface expression of different cytoskeletal proteins on the surface of apoptotic human neutrophils using flow cytometry. In parallel, we studied the intracellular expression of cytoskeletal proteins. Our results indicate that the two INFIL proteins vimentin and lamin B_1, but not the MFAP paxillin, gelsolin, and vinculin nor the microtubule proteins α- and β-tubulin, are expressed on the cell surface of human apoptotic neutrophils.

MATERIALS AND METHODS

Chemicals, agonists, and antibodies

RPMI 1640, HEPES, penicillin/streptomycin (P/S), bovine serum albumin (BSA), *Viscum album* agglutinin-1 (VAA-I), cycloheximide (CHX), lipopolysaccharide (LPS), mouse monoclonal antivinculin antibody (clone hVIN-1), mouse monoclonal antipaxillin antibody (clone PXC-10), mouse monoclonal antigelsolin antibody (clone GS-2C4), mouse monoclonal anti-α-tubulin (clone B-5-1-2), mouse monoclonal anti-β-tubulin (clone 2-28-33), and polyclonal goat anti-human vimentin (clone V4630) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Mouse monoclonal antivimentin (clone V9), rabbit polyclonal antivimentin (clone H84), and goat polyclonal antilamin B_1 (C-20) antibodies were purchased from Santa Cruz Biotechnology (CA). Vimentin antibody (clone 3B4) was obtained from Chemicon (Temecula, CA). Horseradish peroxidase (HRP)-conjugated antibodies, phycoerythrin (PE)-conjugated AffiniPure $F(ab')_2$ fragments, goat anti-*mouse* immunoglobulin G (IgG; $Fab')_2$ fragment-specific and PE-conjugated AffiniPure $F(ab')_2$ fragments, and donkey anti-goat IgG (H$^+$L) were purchased from Jackson Immunoresearch (West Grove, PA). Isotypic antibodies Ms IgG_1 and Ms IgG_{2a} were obtained from PharMingen (Mississauga, Ontario, Canada), and rabbit IgG and goat IgG were from R&D Systems (Hornby, Ontario, Canada). N-benzyloxy-carbonyl-V-A-D-O-methylfluoromethyl ketone (z-VAD-fmk) was purchased from Calbiochem (Pasadena, CA). Granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage (GM)-CSF, and interleukin-4 (IL-4) were purchased from PeproTech Inc. (Rocky Hill, NJ).

Human neutrophils isolation

Neutrophils were isolated from the venous blood of healthy volunteers by dextran sedimentation followed by centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech Inc., Baie d'Urfé, Québec, Canada), as described previously [16, 18]. Blood donations were obtained from informed and consenting individuals according to our institutionally approved procedures. Cell viability (>98%) was monitored by trypan blue exclusion, and the purity (>98%) was verified by cytology from cytocentrifuged preparations colored with the Hema 3 stain set (Biochemical Sciences Inc., Swedesboro, NJ).

Assessment of neutrophil apoptosis

Freshly isolated human neutrophils (10×10^6 cells/mL in RPMI 1640-HEPES-P/S, supplemented with 10% autologous serum) were incubated for 24 h in the presence or absence of neutrophil agonists. Cytocentrifuged samples of neutrophils were prepared using a Cyto-tek® centrifuge (Miles Scientific, Naperville, IL) and processed as documented previously [16, 18, 20]. Cells were examined by light microscopy at 400× final magnification, and apoptotic neutrophils were defined as cells containing one or more characteristic, darkly stained pyknotic nuclei.

Apoptosis was also assessed by flow cytometry following staining with fluorescein isothiocyanate (FITC)-annexin-V, as described previously [19, 21]. Briefly, cells were washed in phosphate-buffered saline (PBS) and resuspended in 100 μl 1× binding buffer (10 mM HEPES/NaOH, pH 7.2, 140 mM NaCl, and 2.5 mM $CaCl_2$), mixed with 2 μl FITC-conjugated annexin-V (Biosource, Montréal, Canada). Cells were gently vortexed and incubated for 15 min at 4°C in the dark. A volume of 400 μl binding buffer was added, and incubation was continued for an additional 15 min in the dark before fluorescein-activated cell sorter analysis (10,000 events) using a FACScan (Becton Dickinson, San Jose, CA).

Cell surface expression of cytoskeletal proteins by flow cytometry

Neutrophils (10×10^6 cells/mL RPMI-HEPES-P/S) were incubated at 37°C, 5% CO_2, in the presence or absence of the indicated neutrophil agonists for 24 h. Cells were harvested in cold PBS and blocked with PBS containing 20% autologous serum for 30 *min* on ice. Cells were washed in PBS and incubated for 30 min on ice with 2 μg/ml mouse monoclonal anticytoskeletal antibodies (antipaxillin, antigelsolin, antivinculin, anti-α-tubulin, anti-β-tubulin, antilamin B_1, or the four antivimentin antibodies). Appropriate isotypic control antibodies were used to compare with the proteins of interest. Cells were washed in PBS and incubated for 30 *min* on ice with FITC-conjugated goat anti-mouse, FITC-conjugated rabbit anti-goat, or FITC-conjugated goat anti-rabbit antibodies. Cell surface expression was analyzed using a FACScan. Results are expressed as a Gmean fluorescence obtained by subtracting the GMean value of the isotypic control from the GMean value obtained with the anticytoskeletal antibody directed against the protein of interest, as the Gmean of the isotypic control varied between the fresh and apoptotic conditions. In other experiments, dual labeling was performed using FITC-annexin-V and antivimentin (V9) or antilamin B_1, followed by their corresponding PE-conjugated antibodies described above.

In some experiments, as apoptotic cells lost CD16 expression, we sorted them from normal apoptotic neutrophils by negative immunomagnetic selection using anti-human CD16-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) as we have published previously [22].

Degradation of cytoskeletal proteins

Neutrophils (10×10^6 cells/mL) were incubated in the presence or absence of agonists for the indicated periods of time, and the expression of cytoskeletal proteins was performed by Western blot as published previously [16, 18]. Briefly, cells were harvested for the preparation of cell lysates in 2× Laemmli's sample buffer. Aliquots corresponding to 250,000 cells were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred from gel to polyvinylidene difluoride membranes. Nonspecific sites were blocked with 1–5% nonfat dry milk or 1% BSA (vinculin and lamin B_1) in Tris-buffered saline-Tween (25 mM Tris-HCl, pH 7.8, 190 mM NaCl, 0.15% Tween-20) for 1 h at room temperature. Membranes were washed and incubated with anti-human cytoskeletal antibodies [mouse monoclonal antivimentin (1:2000 for clones H84, V4630, and V9 or 1:200 for clone 3B4); goat polyclonal antilamin B_1 (1:500); or mouse monoclonal antivinculin (1:150)] overnight at 4°C. After several washes, membranes were incubated with HRP-labeled goat anti-mouse IgG or rabbit anti-goat antibodies (1:20,000 for vimentin and lamin B_1; 1:50,000 for vinculin) for 1 h at room temperature in fresh blocking solution. Bands were revealed with the enhanced chemiluminescence-Western blotting detection system (Amersham Pharmacia Biotech Inc.).

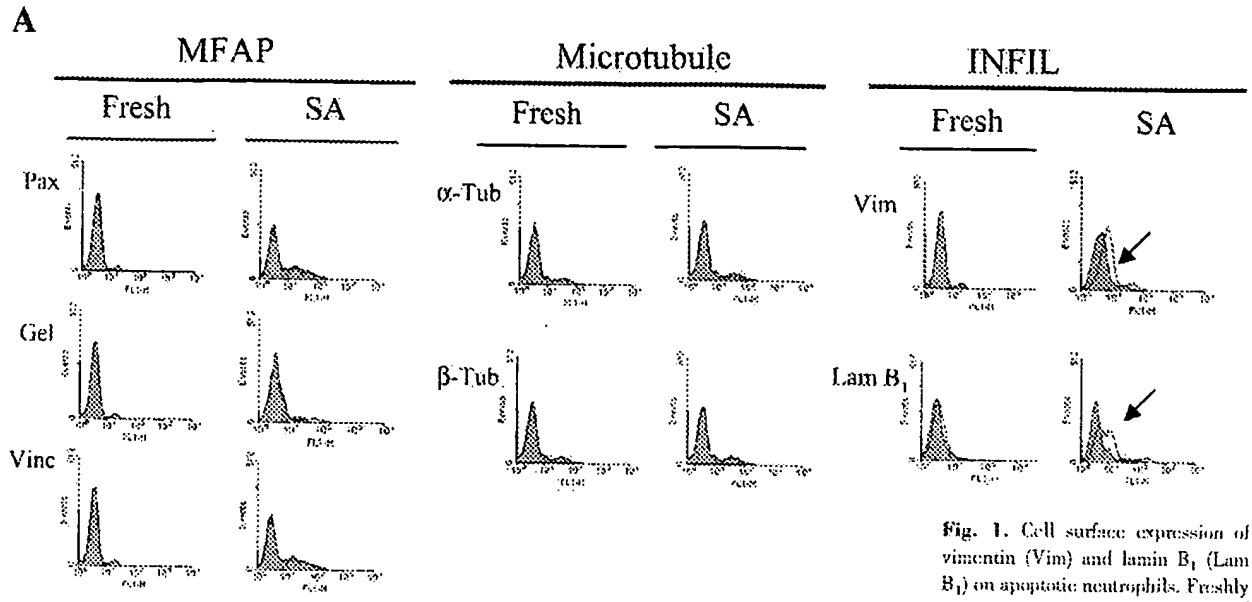
Statistics

Statistical analysis was performed with SigmaStat for Windows Version 2.03 with a one-way ANOVA. Statistical significance was established at $P < 0.05$.

RESULTS

Intermediate filaments are expressed on the surface of apoptotic neutrophils

Knowing that some immune cells express cytoskeletal proteins on their cell surface [6–10], we investigated the possibility that apoptotic neutrophils express some cytoskeletal proteins on their cell surface. As illustrated (**Fig. 1A**) among the different cytoskeletal proteins tested, the three MFAP paxillin, gelsolin, and vinculin, as well as the two major components of microtubules, α-tubulin and β-tubulin, were not expressed on the cell surface of freshly isolated neutrophils or in spontaneous



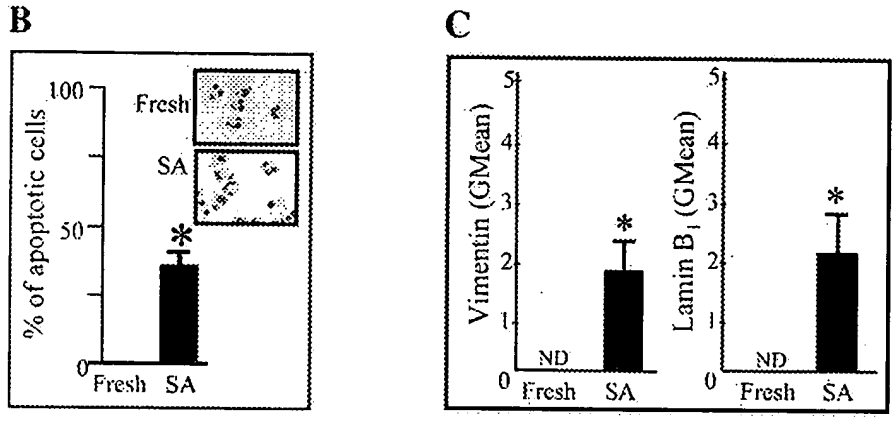


Fig. 1. Cell surface expression of vimentin (Vim) and lamin B_1 (Lam B_1) on apoptotic neutrophils. Freshly isolated human neutrophils (10×10^6 cells/ml) were aged for 24 h or not (Fresh), and cell surface expression of the cytoskeletal proteins (A) was assessed by flow cytometry using the corresponding isotypic control antibody (dark cytograph) as described in Materials and Methods. Results are from one representative experiment of at least five. Arrows indicate cell surface expression (white area). Pax, Paxillin; Gel, gelsolin; Vinc, vinculin; α-Tub, α-tubulin; β-Tub, β-tubulin. The apoptotic rate (B) was assessed by cytology as described in Materials and Methods. (C) A compilation of the Gmeans (mean±SEM, n=5) obtained for the cell surface expression of vimentin (using the V9 clone) and lamin B_1 in fresh and 24-h aged neutrophils. *, $P < 0.05$, by ANOVA. ND, Not determined.

apoptosis (SA). In contrast, we found that the two INFIL vimentin and lamin B_1 proteins were expressed on the cell surface of apoptotic neutrophils. As expected [16, 18, 20], the percentage of neutrophils undergoing SA ranged between 35% and 45% (Fig. 1B). Cell surface expression of vimentin and lamin B_1 on apoptotic cells was reproducible (Fig. 1C).

The tail domain of vimentin is recognized on the surface of apoptotic neutrophils

Vimentin is composed of three domains: the amino-terminal domain (head domain), the central core (rod domain), and the carboxy-terminal domain (tail domain). This is illustrated in **Figure 2A**. Several antivimentin antibodies are known to target specific parts of the protein [8, 23]. Taking advantage of the availability of antivimentin antibodies directed against the different domains, we next investigated which domain of the molecule was detected on apoptotic neutrophils, using four different antibodies. Using three antivimentin antibodies, H84, 3B4, and V9, which are directed against the head, rod, and tail domains of the molecule, respectively, and V4630, an antibody directed against a region overlapping between the rod and tail domains, we found that only V9 stained vimentin on the cell surface of apoptotic neutrophils, as assessed by flow cytometry (Fig. 2B).

Dual labeling of vimentin and lamin B_1 on the cell surface of human neutrophils undergoing SA

To further support that apoptotic neutrophils express intermediate filament proteins on the cell surface, dual labeling was performed using FITC-annexin-V and antivimentin (V9: **Fig. 3B**) or antilamin B_1 (Fig. 3D), followed by their corresponding PE-conjugated antibodies. As illustrated in Figure 3, apoptotic cells, which are annexin-V-positive, express vimentin (Fig. 3B) and lamin B_1 (Fig. 3D, upper/right quadrants). The basic levels of fluorescence are also illustrated in Figure 3, A and C, for corresponding isotypic controls. Apoptotic cells are illustrated in the upper/right quadrants (FITC-annexin-V and cytoskeletal antigen-positive cells). To support that apoptotic neutrophils express intermediate filament proteins on their surface, we sorted apoptotic cells by negative immunomagnetic selection using anti-human CD16-coated magnetic beads, based on the fact that apoptotic neutrophils lose CD16 cell surface expression [24], and we stained them with the antilamin B_1 antibody. As illustrated in the inset of Figure 3, CD16− neutrophils express a high level of lamin B_1 when compared with CD16+ cells.

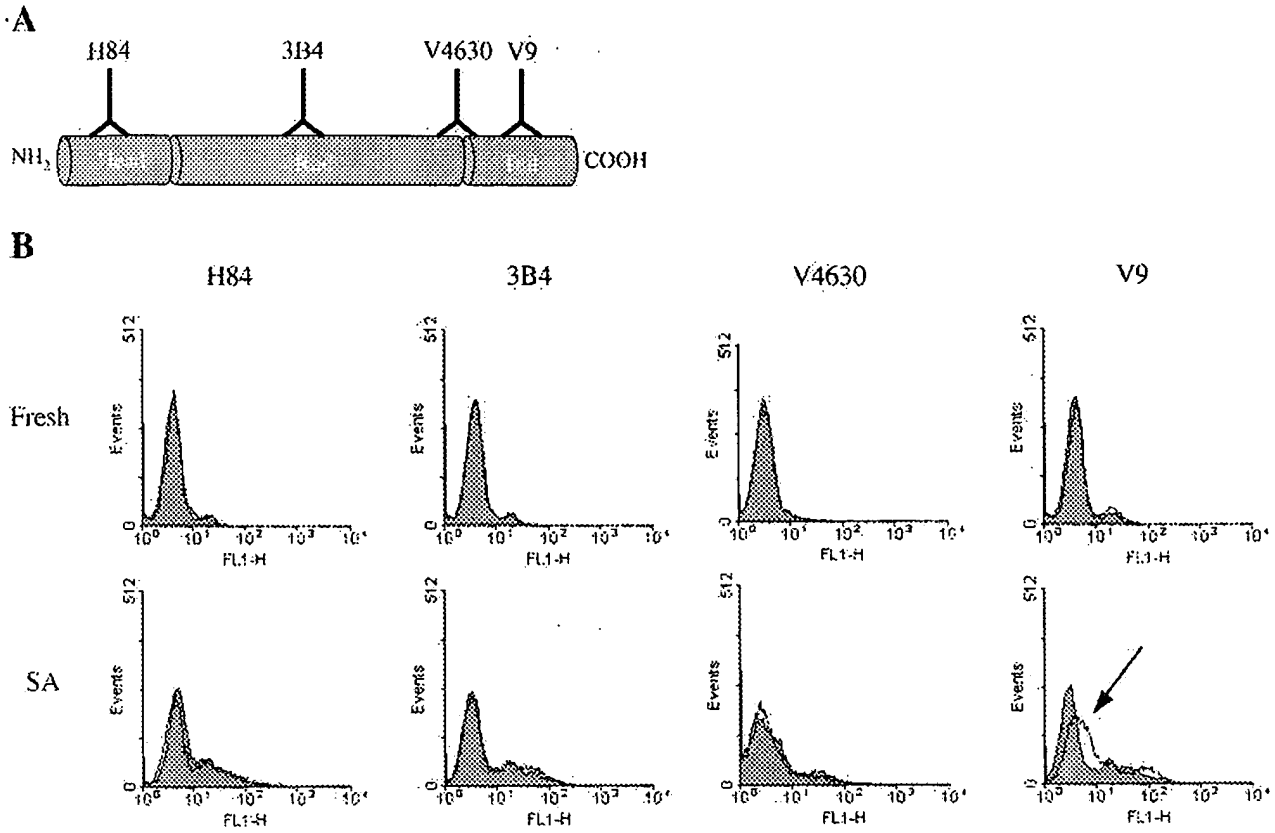


Fig. 2. Cell surface expression of vimentin is only detected with an antibody directed against the tail domain. The specificity of the different antivimentin antibodies used in this study is indicated on the illustrated structure of vimentin (A). (B) Freshly isolated neutrophils (10×10^6 cells/ml) were aged for 24 h (SA) or not (Fresh), and surface expression of vimentin was assessed as described in Materials and Methods. Note that staining was observed only with the V9 clone antivimentin antibody directed against the tail domain of the molecule (arrow). Results are from one representative experiment out of at least five.

Degradation of vimentin in apoptotic neutrophils

As only the V9 antivimentin antibody among the four that were tested detected vimentin on the cell surface of apoptotic neutrophils, we then investigated the intracellular degradation pattern of vimentin obtained with V9 antibody in comparison with the three other antivimentin antibodies. We previously demonstrated that the plant lectin VAA-I was a potent inducer of neutrophil apoptosis, which induced degradation of vimentin via caspases [16, 18]. We therefore studied the degradation of vimentin in SA and in VAA-I-induced apoptosis in parallel. As illustrated in **Figure 4**, all antibodies recognized the native form of vimentin, as well as different fragments known to appear over time when cells undergo apoptosis [19]. The fragments are not related to the sizes of head, rod, or tail fragments of vimentin. It is interesting that addition of the pan caspase inhibitor z-VAD-fmk reversed such degradation, whether or not neutrophils underwent SA or if apoptosis were accelerated by VAA-I. It is clear that the caspase inhibitor partially inhibits the cleavage, suggesting that proteases other than caspases are involved. As others [9], we observed some fragments of vimentin in fresh cells, suggesting that proteolysis occurred during cell isolation.

Role of caspases in the cell surface expression of vimentin and lamin B_1

As degradation of vimentin occurred during SA, addition of caspase inhibitors was previously found to inhibit SA [16, 18], and degradation of this INFIL protein occurred in human neutrophils, we next investigated the potential role(s) of caspases in the cell surface expression of vimentin and lamin B_1, another INFIL protein. As illustrated in **Figure 5**, addition of the pan-caspase inhibitor z-VAD-fmk significantly decreased the cell surface expression of both proteins. The apoptotic rate (means$\pm$SEM, $n=3$) diminished from 56.3 $\pm$ 2.7% to 30.3 $\pm$ 9% when z-VAD-fmk was added.

Cell surface expression of vimentin and lamin B_1 when neutrophil apoptosis is delayed or suppressed

To further associate cell surface expression of vimentin and lamin B_1 with a signal that induced or accelerated neutrophil apoptosis, we decided to investigate intracellular degradation and cell surface expression of these two proteins when apoptosis was delayed or suppressed by different neutrophil agonists. As illustrated in **Figure 6**, cell surface expression of vimentin and lamin B_1 was only observed in SA and not (or rarely, barely detectable) when apoptosis was delayed by G-CSG, GM-CSF, LPS, or IL-4. The apoptotic rate was monitored by cytology and/or flow cytometry after staining with FITC-annexin V and was <45% for SA when the antiapoptotic agents were added to the culture.

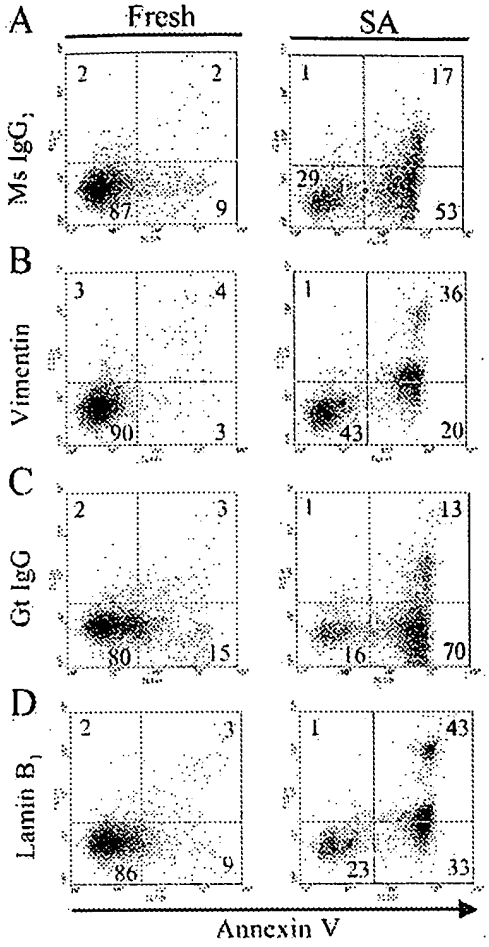
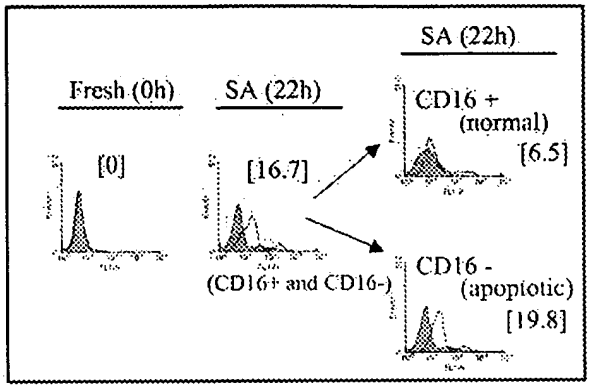


Fig. 3. Dual labeling of vimentin and lamin B_1 on the cell surface of human neutrophils undergoing SA. Freshly isolated human neutrophils (Fresh) were aged for 22–24 h (SA), and dual labeling was performed using FITC-annexin-V and antivimentin (V9; B) or anti-lamin B_1 (D), followed by their corresponding PE-conjugated antibodies, described above as in Materials and Methods. The corresponding basic level of fluorescence (isotypic controls) is illustrated (B, for vimentin; D, for lamin B_1). Numbers in quadrants are the percent of cells from the total population. Apoptotic cells are illustrated in the upper/right quadrants (FITC-annexin and antibody-positive cells). (Inset) Apoptotic cells were sorted by negative immunomagnetic selection using anti-human CD16-coated magnetic beads as described in Materials and Methods. Cell surface expression of lamin B_1 was measured by flow cytometry, and the results are expressed as Cmean of fluorescence (numbers in brackets). Results are from one representative experiment out of three.



Intracellular expression of vimentin and lamin B_1 proteins during neutrophil apoptosis

Next, we decided to study the expression of vimentin and lamin B_1 when neutrophil apoptosis was delayed, as compared with SA, and to verify the role of caspases in the intracellular expression of these two proteins. As illustrated in **Figure 7**, expression of the native forms of vimentin and lamin B_1 was down-regulated drastically in SA when compared with delayed apoptosis. As expected [18], expression of the MFAP vinculin remained relatively stable in the presence or absence (SA) of the antiapoptotic molecules. Knowing that degradation of vimentin occurs by a caspase-dependent mechanism in neutrophils [18], we investigated the role of caspases in the degradation of lamin B_1, in parallel with vimentin in SA and in VAA-1-induced apoptosis. As illustrated in Figure 7, addition of the pan-caspase inhibitor (z-VAD) reversed the cleavage of vimentin and lamin B_1 in SA and in VAA-1-induced apoptosis. The level of expression of vimentin and lamin B_1 in the presence of z-VAD in SA or in VAA-induced apoptosis was similar. However, the levels of vimentin and lamin B_1 were, under all conditions, weaker than the basal levels observed in fresh cells, suggesting that G-CSF, GM-CSF, LPS, and IL-4 did not induce de novo protein synthesis of these proteins but rather prevented their loss of expression. To investigate the potential role of general protein synthesis in the process, we next performed experiments with CHX. As illustrated in **Figure 8**, addition of CHX to the culture during SA or when cells were incubated with the antiapoptotic agents G-CSF, GM-CSF, LPS, or IL-4 was found to reduce the levels of expression of vimentin and lamin B_1, suggesting that protein synthesis is involved in the prevention of degradation of vimentin and lamin B_1.

DISCUSSION

This is the first study demonstrating that human neutrophils can express cytoskeletal proteins on their cell surface. It is interesting that among the seven different proteins we tested, namely, paxillin, gelsolin, vinculin, α-tubulin, β-tubulin, vimentin, and lamin B_1, only the two INFIL proteins, vimentin and lamin B_1, are expressed on the cell surface, suggesting that this is a specific mechanism. Moreover, these proteins are expressed when neutrophils are in apoptosis.

As demonstrated in the present study, vinculin is not expressed on the surface of apoptotic neutrophils. This is in agreement with our previous work, indicating that vinculin is not cleaved during induction of neutrophil apoptosis by VAA-1 [18], tributyltin [17], or methylmercury [19]. However, Melendez et al. [23] have shown that vinculin expression was decreased (a decrease of 33%) during related adhesion focal tyrosine kinase/pyk2-induced apoptosis in cardiomyocytes. In contrast to this latter study, Harrington et al. [25] demonstrated that vinculin was not cleaved during epithelial cell apoptosis

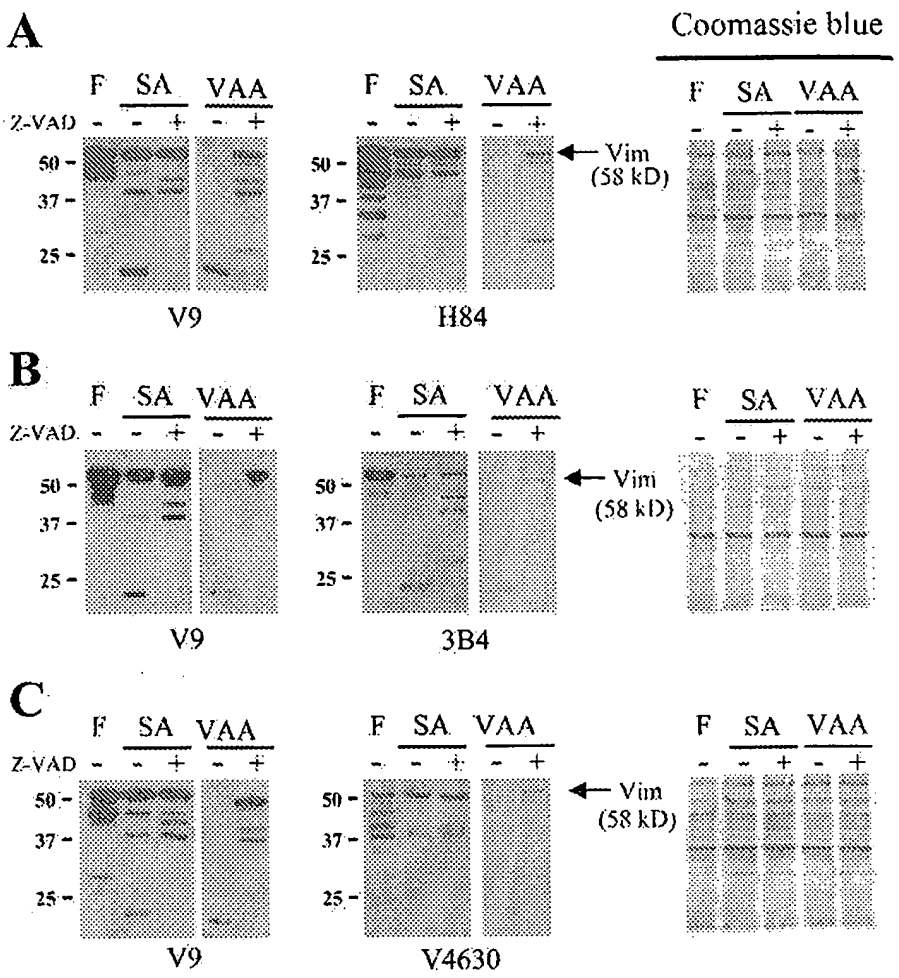


Fig. 4. Comparison of the fragments of vimentin detected by the different antivimentin antibodies and role of caspases in the degradation of intracellular vimentin in spontaneous or VAA-I-induced neutrophil apoptosis. Neutrophils (10×10^6 cells/ml) were incubated with or without (SA) 1000 ng/ml VAA-I for 24 h in the presence (+) or absence (–) of 50 μM pan-caspase inhibitor (Z-VAD). The antivimentin clones are illustrated under each blot. Results are from one representative experiment out of at least three. Membranes were stained by Coomassie blue at the end of the experiments to illustrate equal loading. F, Fresh.

induced by adenosine. In another study [26], expression of vinculin was not modulated by interferon-γ (IFN-γ)-induced apoptosis in the human oropharyngeal epidermoid carcinoma KB cell line. These studies concur with our results, indicating that vinculin remains stable, whether apoptosis is induced or delayed [17–19]. This further demonstrates that cytoskeletal proteins are not all processed in the same way during the apoptotic program. Vinculin is a structural component of focal adhesions, and the reason why it is not cleaved during neutrophil apoptosis is unclear but may be related to the fact that potential cleavage sites are protected by an unknown mechanism. Similar reasoning may also be applied to α- and β-tubulin, as these proteins are not cleaved and are not expressed on the surface of apoptotic neutrophils. However, cleavage of a particular cytoskeletal protein does not necessarily result in its expression on the apoptotic cell surface; this is the case for paxillin and gelsolin, which are cleaved by caspases [16, 18] but are not detected on the surface of apoptotic neutrophils (this report).

INFIL are known to be cleaved into many fragments by caspases during apoptosis. It is not clear if cell surface expression of vimentin and lamin B_1 originates from particular fragmentation products by caspases (or other proteases) or if another mechanism is involved. In various cell types, vimentin is cleaved by caspase-3, -6, -8, and -9 [27–30], whereas lamin B_1 has been found to be cleaved by at least caspase-3 and -6 [31, 32]. The role of caspases in the intracellular degradation of vimentin and lamin B_1 in neutrophils is clear, as treatment with the pan-caspase inhibitor (z-VAD-fmk) partially reversed their cleavage. However, although we demonstrated that addition of z-VAD-fmk reversed the cell surface expression of these two proteins, a direct link with caspase activity in this process is less evident, as addition of this inhibitor also reversed apoptosis, suggesting that cell surface expression of INFIL is also diminished. What is clear is that when apoptosis is suppressed or delayed by G-CSF, GM-CSF, LPS, or IL-4, there are no (or few) INFIL proteins on the neutrophil cell surface. It is of note that even in the presence of the antiapoptotic agents, cleaved vimentin and lamin B_1 are evident, and in some cases, the levels of these cleaved products are as high as in the SA samples. This would argue that the antiapoptotic agents induce synthesis of these proteins, but cleavage still occurs. It is of interest that all of these antiapoptotic agents are known to, themselves, induce de novo protein synthesis in neutrophils [33–35]. In addition, the role of protein synthesis in delaying neutrophil apoptosis has been demonstrated for G-CSF and GM-CSF [33] and for LPS [34]. This is not clear for IL-4 [35]. The cytokine IL-15, like IL-4, is a CD132-dependent cytokine and inhibits the activity of caspase-3 and -8, resulting in a decreased ability to cleave vimentin [36]. Whether this occurs with IL-4 remains to be determined. This raises the possibility that G-CSF, GM-CSF, and LPS prevent the loss of vimentin

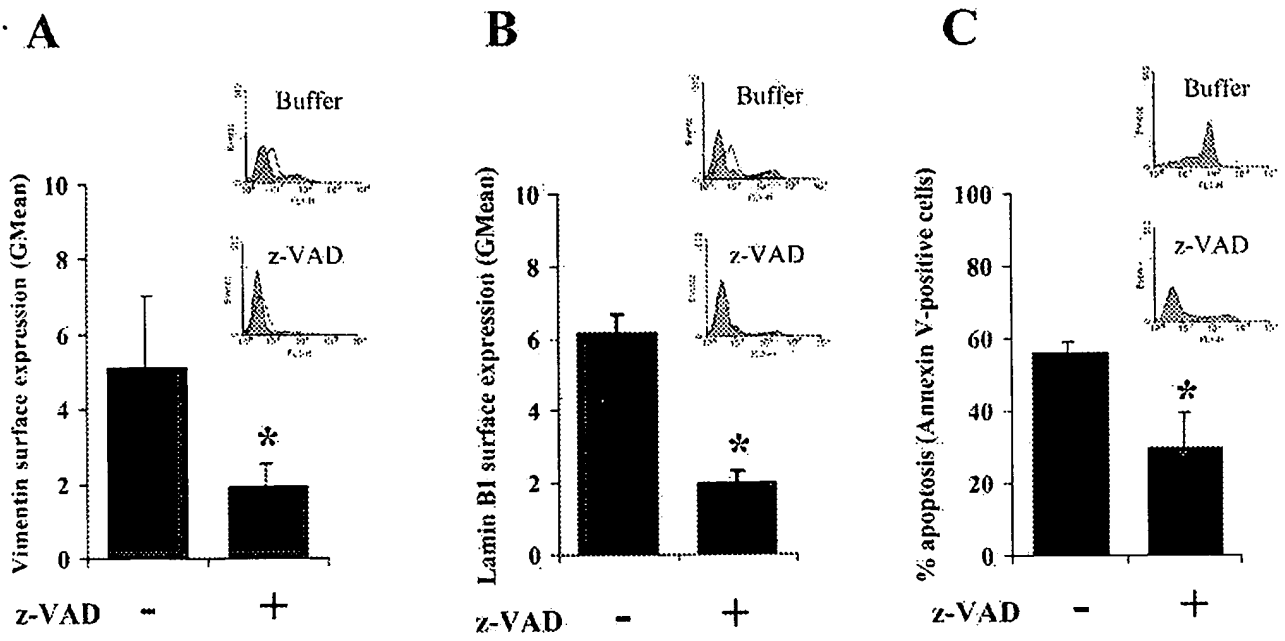


Fig. 5. Potential role of caspases for cell surface expression of vimentin and lamin B_1 in SA. Neutrophils (10×10^6 cells/ml) were incubated for 24 h in the presence (+) or absence (–) of 50 μM pan-caspase inhibitor (z-VAD), and cell surface expression of vimentin (A) and lamin B_1 (B) was assessed in parallel with the same blood donor as described in Materials and Methods. The corresponding apoptotic rate is illustrated (C), as assessed by measuring the number of FITC-annexin-V-positive cells. Results (GMean) are means ± SEM (n=3). *, $P < 0.05$, by ANOVA.

and lamin B_1 expression by inactivating some caspases. It is likely that some proteins requiring continuous synthesis are those that govern caspase activity by an as-yet unknown mechanism. It is noteworthy that the role of protein synthesis in suppression of neutrophil apoptosis is not limited to LPS or cytokines, as dexamethasone also reportedly inhibited apoptosis via continuous protein synthesis [37]. The fact that G-CSF and GM-CSF inhibited neutrophil apoptosis via a protein synthesis-dependent as well as a protein synthesis-independent mechanism indicates the complex mode of action of a given molecule inhibiting neutrophil apoptosis [33].

Antivimentin and antilamin B_1 surface staining is not the result of secondary necrosis, as all cytoskeletal proteins tested (other than INFIL) are not detected on the neutrophil cell

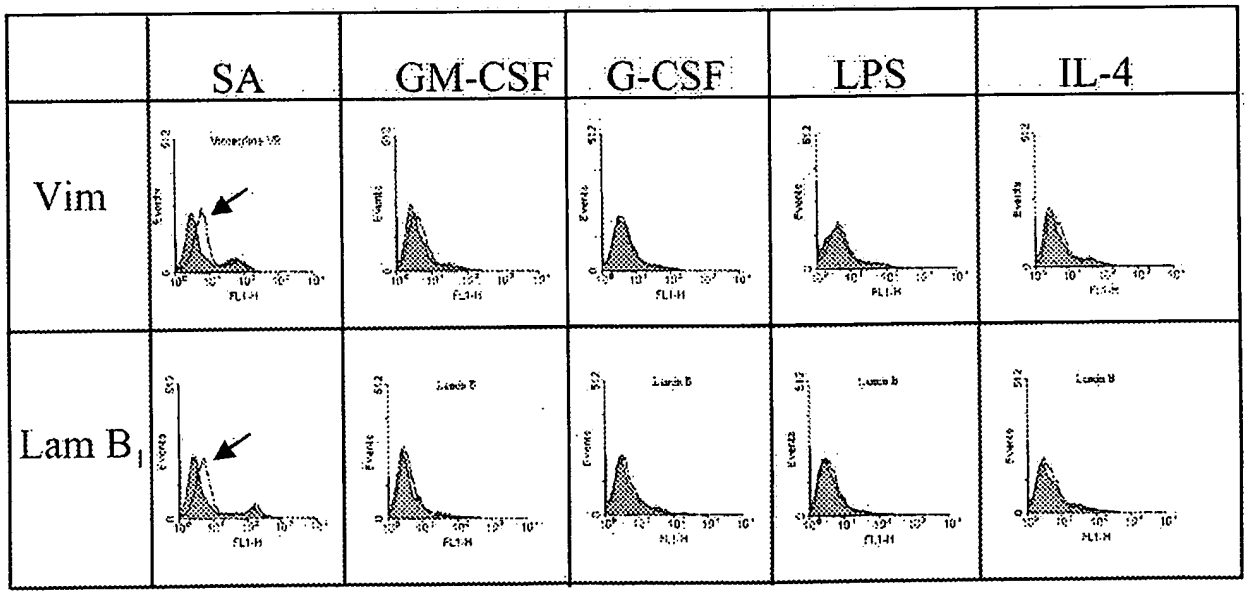


Fig. 6. Cell surface expression of vimentin and lamin B_1 when neutrophil apoptosis is delayed. Neutrophils (10×10^6 cells/ml) were incubated in the presence of buffer (SA), GM-CSF (65 ng/mL), G-CSF (50 ng/ml), LPS (1 μg/ml), or IL-4 (100 ng/mL) for 24 h. Cell surface expression was assessed by flow cytometry as described in Materials and Methods. Results are from one representative experiment out of at least four. Arrows, Cell surface expression of vimentin and lamin B_1 in SA. Note the striking decrease in protein expression when apoptosis is delayed with the antiapoptotic agents.

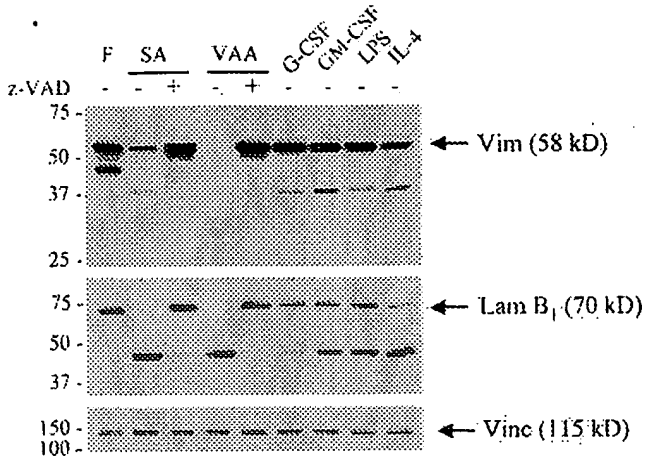


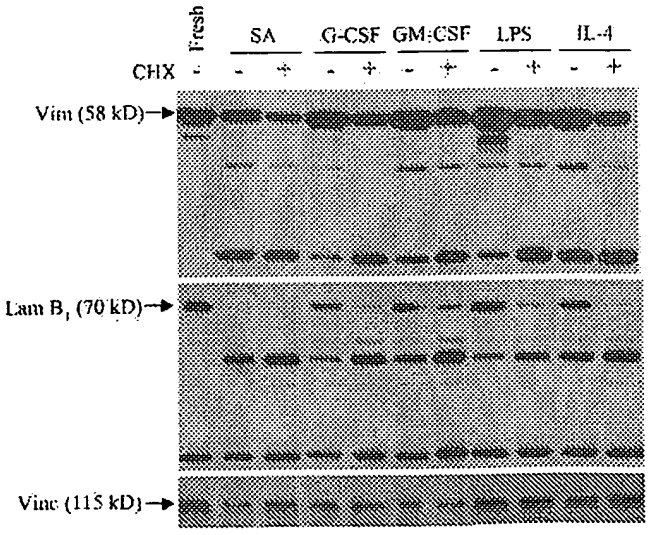
Fig. 7. Intracellular expression of intermediate filaments when neutrophil apoptosis is delayed or accelerated. Neutrophils (10×10^6 cells/ml) were incubated in the presence of buffer (SA) or the antiapoptotic agents G-CSF (50 ng/ml), GM-CSF (65 ng/ml), LPS (1 μg/ml), or IL-4 (100 ng/ml) or with the proapoptotic agent VAA-1 (1000 ng/ml) in the presence (+) or absence (–) of the pan-caspase inhibitor z-VAD-fmk. Cell lysates were prepared, and Western blot experiments were performed as described in Materials and Methods using the V9 antivimentin, the antilamin B_1, or the antivinculin antibodies, as detailed in Materials and Methods. Results are from one representative experiment out of at least three. Note that cleavage of vimentin and lamin B_1 is partially reversed by z-VAD-fmk but that the level of protein expression did not return to the basal levels observed in fresh cells. Vinculin was used to illustrate equal loading, as this cytoskeletal protein is not cleaved during SA or VAA-1-induced neutrophil apoptosis [18].

surface by flow cytometry. Moreover, among four different antivimentin antibodies, only one detected vimentin on the cell surface of apoptotic neutrophils. In addition, cell viability of neutrophils was systematically monitored by trypan blue exclusion. Cell surface expression of vimentin and lamin B_1 appeared to be a relatively late event during neutrophil apoptosis, as 14-h aged cells did not significantly express the protein on their surface (data not shown). Paradoxically, it is known that INFIL are cleaved relatively early during the apoptotic process (few hours), as compared with other cytoskeletal proteins [38]. To ensure that cytoskeletal proteins other than INFIL are not expressed on the neutrophil surface after 24 h, we verified cell surface expression of paxillin, gelsolin, and vinculin on 44-h aged neutrophils and did not observe any of these MFAP, whereas vimentin and lamin B_1 were still detected, and cells were all negative for trypan blue staining (data not shown).

Recently, it has been demonstrated that vimentin is citrullinated during macrophage cell death, suggesting that some of its arginine residues are deiminated to citrulline residues [39]. A few studies have demonstrated that citrullinated vimentin is found in the serum of rheumatoid arthritis patients [3, 40]. Peptidylarginine deiminase (PADI) is an enzyme involved in post-translational modification of peptidylarginine to citrulline in the presence of calcium ions and can change conformation and properties of proteins after their citrullination. Expression of PADI was observed in neutrophils from the synovia of rheumatoid arthritis patients [41, 42]. In the future, it will be interesting to answer whether vimentin (and lamin B_1), detected on the surface of apoptotic neutrophils, is citrullinated.

Neutrophils can generate tyrosyl radicals after activation with phorbol 12-myristate 13-acetate (PMA), IFN-γ, or tumor necrosis factor α [43]. Those radicals act in an autocrine manner by cross-linking to endogenous proteins exposed to the medium. As shown by confocal microscopy, tyrosylated proteins were initially located in patches on the cell surface, internalized, and subsequently degraded. It is interesting that many tyrosylated proteins in neutrophils, including vimentin, have been identified. It was demonstrated that upon cell activation, vimentin was tyrosylated slightly, and the carboxy-terminal part of the protein was phosphorylated at Thr-425. The authors suggested that vimentin phosphorylation was a requirement for its translocation to the plasma membrane. However, they did not demonstrate the presence of vimentin on the neutrophil cell surface.

Using protein kinase C modulators such as PMA and okadaic acid, it was shown that secretion and surface expression of vimentin were regulated by phosphorylation events in macrophages and in a population of endothelial cells [9, 10]. The implication of vimentin phosphorylation for its cell surface expression on apoptotic neutrophils remains to be investigated.



	% apoptosis	
CHX	-	+
SA	59	54
G-CSF	21	52
GM-CSF	22	32
LPS	15	35
IL-4	33	60

Fig. 8. The loss of intracellular intermediate filament expression is prevented by a de novo protein synthesis-dependent mechanism. Neutrophils (10×10^6 cells/ml) were incubated for 24 h with buffer (SA) or the antiapoptotic agents in the presence (+) or absence (–) of CHX (2 μg/ml). Western blot experiments were performed as described in Materials and Methods. Results are from one representative experiment out of at least three. Note that for all antiapoptotic agents, the levels of expression of vimentin and lamin B_1 are decreased in the presence of CHX. The corresponding apoptotic rates, as assessed by cytology, are illustrated in the table on the right.

However, activation of neutrophils by the antiapoptotic agents GM-CSF, G-CSF, LPS, and IL-4, which are known to induce phosphorylation events in neutrophils, maintained intracellular INFIL expression, probably via de novo protein synthesis, but did not lead to vimentin and lamin B_1 cell surface expression. As assessed by immunoblotting, we failed to detect the presence of vimentin and lamin B_1 in the extracellular milieu after neutrophil activation with these agents (data not shown).

Antilamin B autoantibodies are detected in many autoimmune diseases, including autoimmune liver diseases, rheumatoid arthritis, and systemic lupus erythematosus (SLE) [44]. Dieude et al. [5] have demonstrated that antilamin B autoantibodies from SLE patients do not bind to the surface of apoptotic blebs in Jurkat T cells and human umbilical vein endothelial cells. Using confocal microscopy, they demonstrated that lamin B was not present on the surface of these blebs but rather remains buried within the blebs, rendering them inaccessible to external antilamin B_1 antibodies. However, unlike these cells, neutrophils do not form typical blebs when they undergo apoptosis.

Boilard et al. [8] determined the specificity of various antivimentin antibodies. In their study, they determined that the goat polyclonal V4630 antivimentin antibody recognized the rod and tail domain of the protein. They found that rod and tail domains of vimentin were exposed on the cell surface of human apoptotic T lymphocytes, as the V9 and V4630 antibodies recognized the protein on the cell surface. Herein, we demonstrated that only the antibody directed against the tail domain of vimentin-stained apoptotic neutrophils, suggesting that unlike T lymphocytes, apoptotic neutrophils exposed only a part of the tail domain of vimentin on the surface.

Although we only detected the presence of INFIL on the surface of apoptotic neutrophils, we cannot rule out the possibility that some of the proteins we tested (as well as other cytoskeletal proteins) are expressed on the cell surface, as it is possible that the antibodies we used in this study may not recognize particular epitopes on the neutrophil surface. Although the antibodies used to detect gelsolin, paxillin, vimentin (clone V9), and lamin B_1 are all directed against the carboxy-terminal end of proteins, only those directed against vimentin and lamin B_1 stained apoptotic neutrophils.

Xu et al. [10] suggested that secreted vimentin could play a role in mediating the movement of circulating blood cells across the endothelium, a process in which activated macrophages and activated platelets participate. Knowing that cell surface and secreted vimentin are involved in various biological functions, it is tempting to speculate that the protein exposed on the surface of apoptotic neutrophils could serve as an "eat me" signal, helping phagocytes to recognize and ingest apoptotic neutrophils, but this remains to be demonstrated. In addition, lamin B_1 could also be involved in this process. As previously mentioned, the antilamin B_1 antibody we used is also directed against the carboxy-terminal part of the protein. However, in contrast to vimentin, antibodies directed against different parts of lamin B_1 are not well characterized. In addition to the potential role in apoptotic neutrophil elimination, neutrophils may represent an important source of cytoskeletal autoantigens for the development of autoantibodies directed against cytoskeletal proteins, normally sequestered inside the cell, at least for the two INFIL proteins vimentin and lamin B_1.

The results of this study establish for the first time that apoptotic neutrophils express some cytoskeletal proteins on their surface. Among different proteins that we have tested, including members of the three classes of cytoskeletal filaments, namely, vinculin, paxillin, and gelsolin (microfilaments), vimentin and lamin B_1 (INFIL), and α- and β-tubulin (microtubules), only those of the INFIL are detected on apoptotic neutrophils.

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